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# Extracellular vesicles targeting non-parenchymal cells: the therapeutical effect on liver fibrosis

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## ABSTRACT

Liver fibrosis is the formation of a fibrous scar due to chronic liver disease including viral hepatitis, alcohol and non-alcoholic fatty liver disease. Without treatment, it will develop into cirrhosis and hepatocellular carcinoma. Up to now, there is no effective way to cure liver fibrosis. Extracellular vesicles (EVs) are biological nanoparticles with potential to be therapeutical agents or delivery tools. A lot of studies have demonstrated the therapeutical effect of EVs on liver fibrosis. In this review, we mainly pay attention to roles of liver non-parenchymal cells in pathology of fibrosis, the basic information about EVs and therapeutical effect on liver fibrosis of EVs when they act on non-parenchymal cells.

## INTRODUCTION

Liver disease is a significant cause of mortality, accounting for 2 million deaths annually and representing 4% of all global deaths (1 in every 25 deaths).[1] The primary causes of cirrhosis worldwide include viral hepatitis, alcohol abuse and non-alcoholic fatty liver disease (NAFLD).[1] Chronic liver injury invariably leads to liver fibrosis, a common and potentially life-threatening condition with global prevalence. Without intervention, liver fibrosis progresses to cirrhosis and eventually hepatocellular carcinoma, resulting in high mortality rates and substantial economic burdens.

Hepatic fibrosis occurs when fibrous scar tissue forms due to the accumulation of extracellular matrix (ECM) proteins, primarily cross-linked collagens type I and type III, replacing damaged normal liver tissue. Throughout its development and regression, non-parenchymal cells within the liver play crucial roles.[2] Non-parenchymal cells mainly include hepatic stellate cells (HSCs), liver sinusoidal endothelial cells (LSECs) and hepatic macrophages (also called Kupffer cells (KC)), and these cells only account for 6.5% of the liver volume, but 40% of the total number of liver cells. They release various substances to regulate or assist the functions of hepatocytes or neighbouring non-parenchymal cells. Consequently, therapies targeting these cells have gained popularity as potential strategies to alleviate liver fibrosis. Extracellular vesicles (EVs), biological nanoparticles released by mammalian cells that encapsulate a lipid bilayer, can serve as therapeutic agents or delivery tools.[3] With their ability to be engineered for targeted delivery, EVs are being explored for their therapeutic effects in liver fibrosis treatment.

This review will primarily focus on the involvement of liver non-parenchymal cells in fibrosis and the use of EVs in targeting these cells.

## NON-PARENCHYMAL CELLS IN LIVER FIBROSIS

### Hepatic stellate cells

HSCs normally exhibit a quiescent phenotype and reside in the space of Disse, functioning as pericytes. Quiescent HSCs are characterised by their storage of retinoids, and depletion of retinoids on HSC activation can lead to systemic vitamin A deficiency.[4] HSC activation is a dynamic process that occurs in response to injury. It involves a series of phenotypic changes, including ECM production. Activated HSCs undergo loss of lipid droplets, express contractile fibres, transition from an adipogenic to a myofibroblast phenotype, increase cell proliferation and chemotaxis and signal to attract leucocytes. Additionally, matured rough endoplasmic reticulum develops to support the production of ECM fibres and matrix remodelling enzymes.[5]

For instance, activated HSCs and myofibroblasts produce various members of the lysyl oxidase (LOX) family, such as LOX, LOXL1, LOXL2, LOXL3 and LOXL4. These extracellular copper-dependent enzymes catalyse the cross-linking of structural ECM components in fibrotic organs, including the liver. Inhibition of the LOX family has been shown to suppress fibrosis progression and accelerate its reversal in rodent models.[6 7] Moreover,

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during HSC activation, ribosomal proteins, proteins associated with cell cycle control and migration and other factors increase, as observed in an HSC activation model using immortalised human LX-2 HSCs and fetal bovine serum.[8]

All these cellular processes contribute to the local accumulation of ECM, ultimately leading to scar formation in injured areas of the liver. If the profibrogenic triggers cease, fibrosis can regress. Activated HSCs can either revert to a quiescent phenotype or undergo senescence or apoptosis.[9] Reversion of activated HSCs to a quiescent phenotype involves the elimination of profibrotic conditions such as CCl4 or alcohol. However, the phenotype of reactivated HSCs remains distinct from that of quiescent cells. While the expression of fibrogenic genes (eg, collagen-α1, α-smooth muscle actin (α-SMA)) returns to baseline, the expression of genes characteristic of quiescent HSCs (eg, glial fibrillar acidic protein, adiponectin receptor 1, D site of albumin promoter binding protein) is not fully restored.[10]

## Kupffer cells

Hepatic macrophages play crucial roles in various aspects of liver function, including systemic homeostasis, infection response, initiation of inflammatory reactions to liver injury, fibrosis progression, wound healing promotion and resolution of liver fibrosis.[11] These macrophages can be categorised into two groups: liver-resident cells known as KCs and monocyte-derived macrophages originating from the bone marrow. In the context of liver fibrosis, hepatocytes that are damaged release damage-associated molecular patterns (DAMPs), which interact with KCs, triggering injury-induced inflammation and subsequent recruitment of KCs to the site of injury. At this site, they are activated and polarised through cytokines and chemokines.[12] The activation of KCs leads to the secretion of proinflammatory and fibrogenic cytokines, promoting the activation of HSCs and ultimately resulting in liver fibrosis.

KCs can interact with HSCs, which are recognised as key effector cells and sources of myofibroblasts, as well as producers of extracellular fibrillar collagen, leading to remodelling of the immune microenvironment and ECM. Initially, KCs secrete cytokines and chemokines such as transforming growth factor (TGF)-β, tumour necrosis factor (TNF)-α, interleukin (IL)-1β and C-C motif chemokine 2 to stimulate HSCs. TGF-β, in particular, plays a significant role in fibrosis, and its deletion or blockade has been shown to ameliorate liver fibrosis in mice by inhibiting the expression of collagen I and α-SMA in HSCs.[13 14] KCs also produce IL-1β and TNF-α, which enhance myofibroblast proliferation through nuclear factor kappa B signalling pathways. Activin-A, another member of the TGF-β superfamily, is upregulated in alcoholic cirrhosis and non-alcoholic steatohepatitis (NASH), and its increased expression in KCs induces a fibrogenic phenotype in HSCs by upregulating TNF-α and TGF-β1.[15] Additionally, high expression of stimulator of interferon genes in KCs in patients with NAFLD contributes to enhanced activation of HSCs and proinflammatory responses in hepatocytes through increased secretion of TGF-β1, TNF-α and IL-1β.[16] Apart from cytokine and chemokine production, resident liver macrophages and infiltrating Gr-1(+) myeloid cells can control the release of mitochondrial DAMPs from dying hepatocytes, thereby activating HSCs. Thus, modulating the phagocytic function of KCs or targeting the release of mito-DAMPs from hepatocytes holds potential as an antifibrotic strategy.[17]

During the regression stage of fibrosis, KCs express matrix metalloprotein-9 (MMP9) and TNF-related apoptosis-inducing ligand, facilitating apoptosis of myofibroblasts,[18] and KCs participate in the removal of dead/dying cells through efferocytosis.[19] This mechanism plays a significant role in regulating fibrotic outcomes. Conversely, phagocytosis of apoptotic cells by KCs promotes matrix metalloproteinase (MMP) expression and ECM degradation in rodent models of fibrosis regression. In NASH, necroptotic hepatocytes express CD47, a 'don't-eat-me' ligand that helps them avoid clearance by KCs. Therefore, blocking the CD47-SIRPα axis could be a strategy to reduce the accumulation of necroptotic hepatocytes and accelerate fibrosis resolution.[20]

## Liver sinusoidal endothelial cells

LSECs are specialised endothelial cells (EC) in the liver with distinct morphology and functions. Morphologically, LSECs feature fenestrae (pores) of 100–150 nm size, enabling exchange between the sinusoidal blood and Disse space.[21] Functionally, LSECs exhibit a high capacity for endocytosis due to various endocytosis receptors such as collagen-α-chain/mannose receptor, hyaluronan/scavenger receptor and FccIIb2 receptor.[22]

During sustained hepatic injury, LSECs undergo phenotypic changes and lose their protective properties, contributing to liver fibrosis progression. In response to liver injury, LSECs transform into capillarised LSECs characterised by the loss of fenestrae and LSEC markers. This process is facilitated by upregulated endothelial Notch signalling, which inhibits endogenous nitric oxide synthase (eNOS) transcription and leads to Delta-like ligand 4 overexpression.[23 24] Vascular cell adhesion molecule 1 expression activation further promotes LSEC capillarisation by activating YAP1 protein.[25] Additionally, vascular endothelial growth factor (VEGF) secreted by HSCs and hepatocytes helps maintain LSEC phenotype through nitric oxide (NO)-dependent and NO-independent pathways. In detail, VEGF stimulates NO release from eNOS in LSEC. NO in turn acts through soluble guanylate cyclase (sGC), conversion of GTP to cGMP and stimulation of protein kinase G, which can then phosphorylate protein targets and maintain LSEC phenotype.[26 27] Single-cell transcriptomics studies have revealed the spatial distribution of heterogeneous liver ECs in normal and cirrhotic mouse livers. Capillarisation-associated genes and ECM genes were found to be upregulated in LSECs



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of zone 3 in cirrhotic mice, while expression of endocytic receptors decreased. Transcription factors involved in NO production, such as Kruppel-like factor (Klf)2, Klf4 and activator protein 1, were downregulated.[28] As a result of phenotypic transition, LSECs lose their antifibrotic capacities and directly or indirectly participate in fibrosis. They secrete ECM components in response to TGF-β1, contributing to fibrosis initiation. Capillarisation occurs prior to HSC activation and disrupts metabolic interchange while exacerbating hepatocyte hypoxia.[29 30] Furthermore, dedifferentiated LSECs play a role in generating the inflammatory milieu and altering intrahepatic immunity, promoting fibrosis.[31]

Fibrosis is considered a dynamic and reversible process termed fibrosis resolution/regression when the underlying causes are eliminated. Coculturing differentiated LSECs with activated HSCs and using an sGC activator to maintain LSEC differentiation can lead to the reversion of activated HSCs back to a quiescent state. sGC participates in VEGF stimulation of NO-dependent signalling with increased eNOS expression to promote hepatic EC-dependent regression of liver fibrosis.[27 32] Moreover, LSECs coordinate immune cell recruitment and actively participate in the endocytosis and clearance of denatured collagen-α chains.[33 34] In summary, LSECs play a crucial role in fibrosis resolution.

During development of liver fibrosis, non-parenchymal cells play an important role in many ways (figure 1).

## BIOGENESIS, SECRETION AND CELLULAR ENTRY OF EVS

EVs are small nanoparticles released by mammalian cells, enclosed within a lipid bilayer. They can be categorised into exosomes, microvesicles and apoptotic bodies based on their size, morphology, biogenesis, membrane composition and cargo content.[35] The life cycle of EVs involves biogenesis, secretion and cellular entry (figure 2).

EV biogenesis begins with the inward budding or endocytosis of the plasma membrane (PM) into the cell, resulting in the production of an intraluminal vesicle (ILV) that contains cytoplasmic constituents.[36] As ILVs accumulate, multivesicular bodies (MVBs) are generated; MVBs have two possible fates: fusion with the PM or fusion with lysosomes.[37] In the case of fusion with the PM, small GTPases Rab27a and Rab27b assist MVBs in docking with the PM. Secretory MVBs then bind to soluble N-ethylmaleimide-sensitive factor attachment protein receptors on the membrane.[38 39] Eventually, they

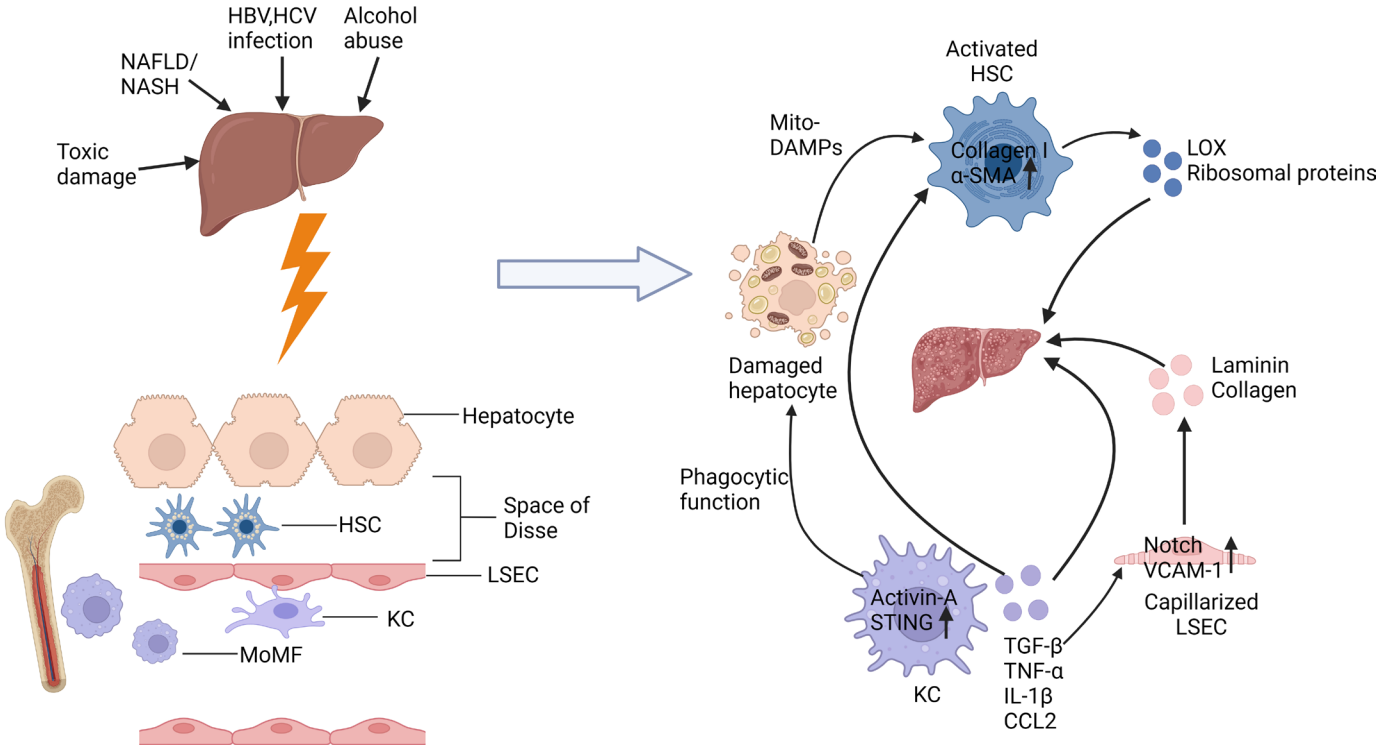


**Figure 1** Phenotypic changes in non-parenchymal cells during development of liver fibrosis. In response to chronic stimuli such as alcohol abuse, viral infection, NAFLD/NASH and toxic damage, liver loses its normal structure and non-parenchymal cells including HSCs, KCs and LSECs undergo phenotypic changes. HSCs and KCs are in activation and LSECs become capillarised with loss of fenestrae and markers followed by release of fibrogenic stimuli such as TGF-β, LOX and so on, which play important roles in the development of liver fibrosis. CCL2, C-C motif chemokine ligand 2; HBV, hepatitis B virus; HCV, hepatitis C virus; HSC, hepatic stellate cell; IL, interleukin; KC, Kupffer cell; LOX, lysyl oxidase; LSEC, liver sinusoidal endothelial cell; Mito-DAMP, mitochondrial damage-associated molecular pattern; MoMF, monocyte-derived macrophage; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; SMA, smooth muscle actin; STING, stimulator of interferon gene; TGF, transforming growth factor; TNF, tumour necrosis factor; VCAM-1, vascular cell adhesion molecule 1. (Created with bioRender.com)



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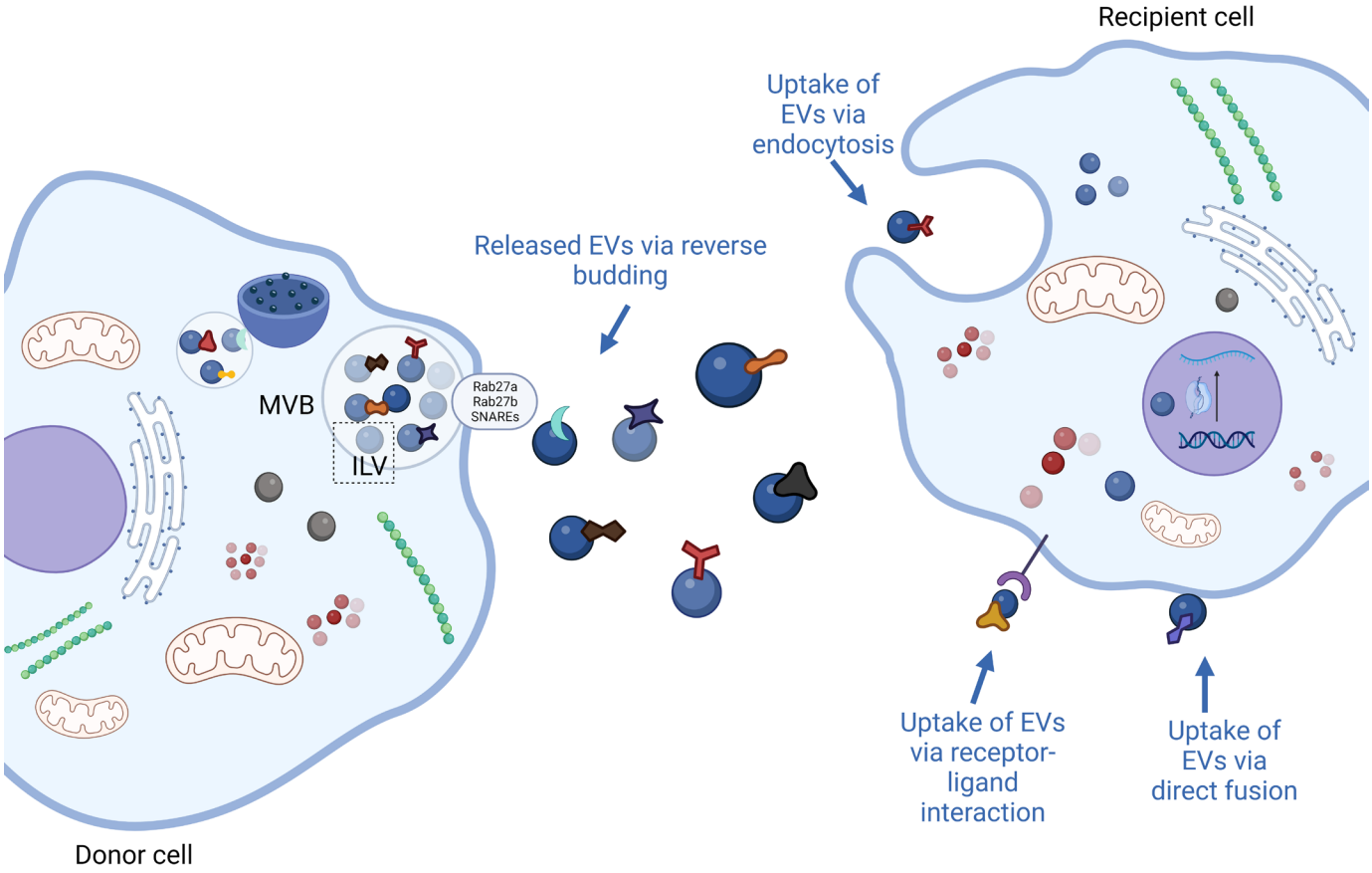


**Figure 2** Biogenesis, release and uptake of EVs. EVs are formed by inward budding or endocytosis of the plasma membrane (PM) into the cell. After that, EVs mature gradually and finally are released to extracellular space in the EV generation pathway. Then, EVs enter target cells and interact with them through various mediators such as surface receptors and signalling events in order to influence phenotypes of recipient cells. EVs, extracellular vesicles; ILV, intraluminal vesicle; MVB, multivesicular body; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor. (Created with bioRender.com)

fuse with the PM, releasing EVs into the extracellular space.

Once EVs are released into the extracellular space, they can interact with recipient cells and influence their phenotypes. To enter target cells, EVs first need to dock at the PM, which is facilitated by the activation of surface receptors and signalling events. Then they can be uptaken by recipient cells via endocytosis, receptor-ligand interaction or direct fusion. Various mediators of these interactions have been identified, including tetraspanins, integrins, lipids, lectins, heparan sulfate proteoglycans and ECM components.[40 41] Once internalised or fused with target cells, EVs deliver signals to recipient cells either by binding and activating receptors on their surface or by delivering cargo molecules into the recipient cells.[42 43] Eventually, EVs are targeted to lysosomes, where the proteins and lipids they carry are degraded, providing a relevant source of metabolites to the recipient cells.[44]

## THERAPEUTIC POTENTIAL OF EVS

EVs show a therapeutic potential in liver fibrosis. They can be therapeutical agents to improve liver fibrosis directly and they also can deliver drugs to specific cells with or without modification (figure 3).

### EVs as therapeutical agent

EVs derived from stem cells or tumour cells can exhibit organotropic or tissue-tropic properties, making them potentially effective for therapeutic purposes in preclinical models of liver disease or cancer. Stem cell-derived EVs, particularly those derived from hepatic stem-like cells (human liver stem cells (HLSCs)), have shown promising results in improving liver function and reducing signs of liver fibrosis and inflammation in mice with liver disease induced by a methionine-choline-deficient diet.[45]

Mesenchymal stem/stromal cells (MSCs) and their EVs have also been extensively studied for liver dysfunction therapies. MSC-derived EVs contain bioactive molecules such as proteins, lipids and RNA. In animal models of acute liver failure/acute liver injury, bone marrow-derived MSC exosomes have been found to attenuate hepatic inflammation, necrosis and oxidative stress and promote hepatocyte proliferation. They achieve this by downregulating proapoptotic proteins Bax and cleaved caspase-3, as well as upregulating autophagy markers.[46 47] In chronic liver diseases, MSC exosomes have demonstrated the



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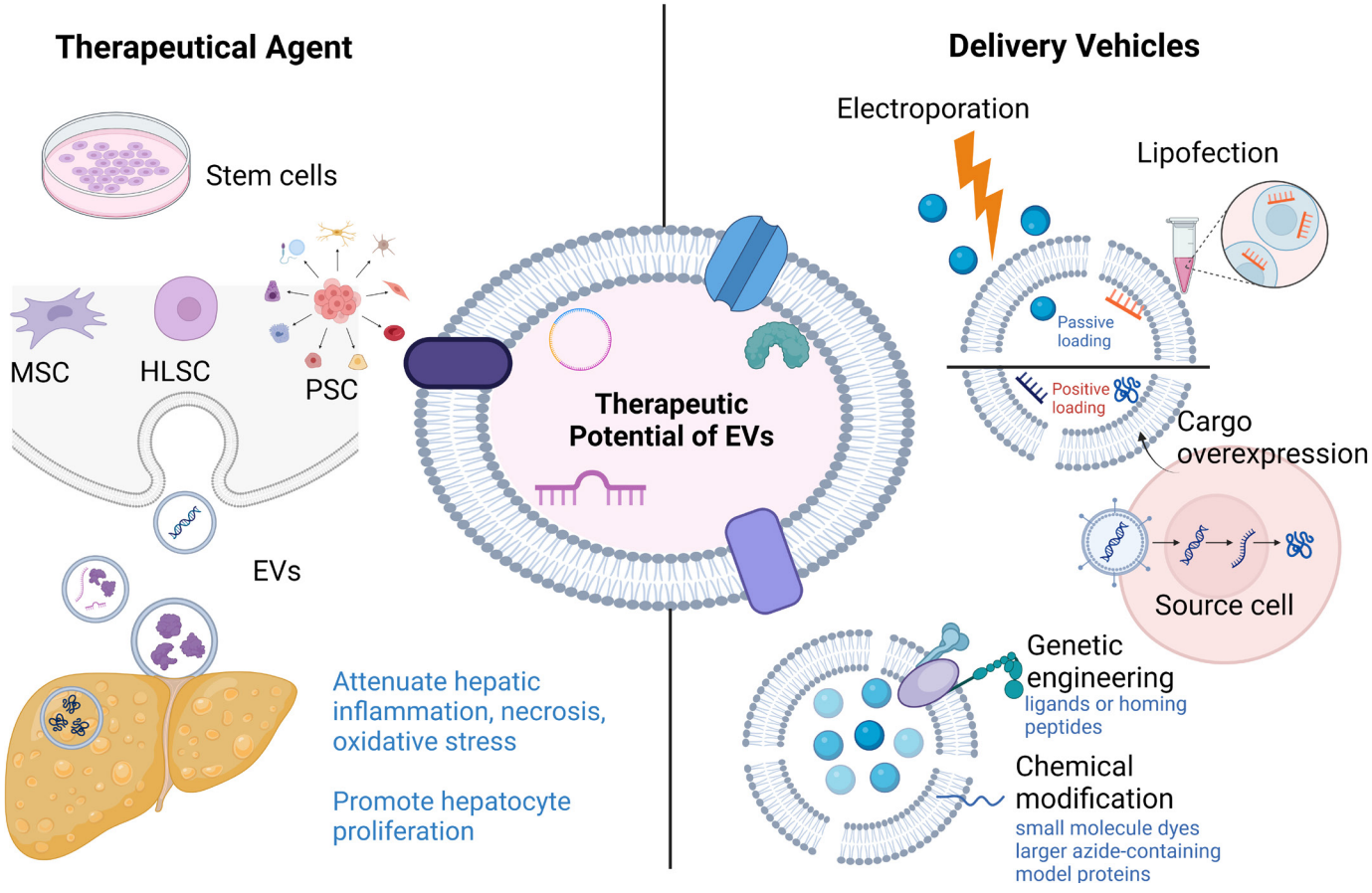


**Figure 3** Therapeutic potential of EVs. EVs can be used as therapeutical agent or delivery vehicles. As therapeutical agent, EVs are always from stem cells and they can attenuate hepatic inflammation, necrosis and oxidative stress and promote hepatocyte proliferation. As delivery vehicles, EVs loading drugs with genetic or chemical modification target liver to improve fibrosis. EVs, extracellular vesicles; HLSC, human liver stem cell; MSC, mesenchymal stem/stromal cell; PSC, pluripotent stem cell. (Created with bioRender.com)

ability to alleviate liver injury, inhibit fibrotic pathways (eg, STAT3/Bcl-2/Beclin pathway), reduce α-SMA expression associated with liver fibrosis and alleviate autoimmune hepatitis-related liver necrosis and inflammatory markers such as TNF-α, IL-17 and IL-1β.[48–50]

## EVs as delivery vehicles

EVs possess favourable characteristics for the use as delivery vehicles for biologically active molecules, including chemotherapy drugs, small-interfering RNA (siRNA) or immunologically active agents.[51] Cargo loading into EVs can be achieved through passive loading methods, such as electroporation and lipofection, which involve incorporating the drug inside the exosomes based on size differences. Active loading methods involve fusing transmembrane proteins or RNA molecules with EV cargo during their biogenesis.[52 53]

EVs offer several advantages as delivery vehicles, including versatility, excellent biocompatibility and a tendency to accumulate in the liver. Moreover, EVs can be isolated from various sources, not just cells. They can be obtained from plasma, different tissues, milk or even plants. For example, bovine milk EVs can be easily engineered to carry small non-coding RNAs that target hepatic (HepG2) and intestinal (Caco-2) cell lines.[54] In summary, EVs as delivery vehicles can enhance the stability of hydrophobic drugs and small molecules while improving their bioavailability and reducing off-target side effects.

## Surface engineering for targeted delivery

Surface engineering of EVs is a strategy used to enhance their targeting potential, and it involves two main approaches: genetic engineering and chemical modification[54 55] (table 1).

### Genetic engineering

Genetic engineering offers a convenient way to impart new properties to exosomes. The process involves fusing ligands or homing peptides with transmembrane proteins expressed on the surface of exosomes. One commonly used transmembrane protein for this purpose is LAMP-2B, a member of the lysosome-associated membrane protein family. LAMP-2B predominantly localises to lysosomes and endosomes, with a small fraction present on the cell surface. Its N-terminus is exposed on the surface of exosomes, allowing targeting sequences to be attached to it.[56] Various peptides have been used for different types of targeting. For example, the rabies virus glycoprotein



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Table 1 Therapeutic effects of surface engineering EVs

| Modification types | Modifier types | Therapeutic effect | Refs |
|---|---|---|---|
| Genetic engineering | Rabies virus glycoprotein (RVG) peptide | Target the central nervous system. | 56 |
| | αγ integrin-specific iRGD peptide | Deliver doxorubicin (DOX) to integrin-positive breast cancer cells. | 58 |
| | NSCLC-homing tLyP-1 peptide | Deliver siRNA to human NSCLC cells. | 59 |
| Chemical modification | RGE peptide (RGERPPR) | Enable exosomes to penetrate the BBB and target glioma. | 62 |
| | Peptide c(RGDyK) | Enable exosomes to penetrate the BBB and suppress inflammatory responses and cellular apoptosis. | 63 |
| | SIRPα antibodies | Interfere the interaction between CD47 and SIRPα. | 63 |
| | DSPE-PEG | Deliver PTX to lung cell lines. | 64 |
| | RVG peptide ligand | Enhance MSC-derived exosomes binding to the cortex and hippocampus. | 65 |

BBB, blood-brain barrier; EVs, extracellular vesicles; MSC, mesenchymal stem/stromal cell; NSCLC, non-small cell lung cancer; PTX, paclitaxel.

(RVG) peptide facilitates neurospecific exosomes to target the central nervous system.[57]The αγ integrin-specific iRGD peptide aids in the delivery of doxorubicin to integrin-positive breast cancer cells in vitro and in vivo.[58] The tLyP-1 peptide, a non-small cell lung cancer (NSCLC)-homing peptide, targets neuropilin-1 (NRP1) and neuropilin-2 (NRP2) receptors, enabling the delivery of siRNA to human NSCLC cells.[59] Apart from peptides, LAMP-2B can also be fused with targeting proteins or antibody fragments to display antibodies on exosomes.[60] Additionally, a glycosylation motif (GNSTM) can be added to the N-terminus of peptide-LAMP-2B fusions to enhance peptide stability.[61] These strategies provide viable approaches for creating novel targeted therapeutics using engineered exosomes, allowing for specific delivery to desired cell types or tissues.

### Chemical modification

Chemical methods are commonly employed to modify the surface of exosomes. Click chemistry enables the attachment of both small molecule dyes and larger azide-containing model proteins to the exosome surface. For instance, the RGE peptide (RGERPPR) can be conjugated to exosomes using a cycloaddition reaction with sulfonyl azide, enabling them to penetrate the blood-brain barrier (BBB) and target glioma.[62] A bio-orthogonal click reaction can conjugate the peptide c(RGDyK) to the exosome surface, providing them with the ability to penetrate the BBB, suppress inflammatory responses and prevent cellular apoptosis.[63] By clicking azide-modified exosomes with dibenzocyclooctyne-derivatised SIRPα antibodies, the exosomes can serve as immune checkpoint blockers to interfere with the interaction between CD47 and SIRPα.[63] Another approach to chemical modification involves the insertion of amphipathic molecules into the lipid bilayer of exosomes. DSPE-PEG can be linked with aminoethyl anisamide, a high-affinity sigma-selective ligand, resulting in its accumulation in the exosome membrane. This modification leads to increased uptake of AA-modified exosomes in lung cell lines, facilitating targeted delivery of paclitaxel and enhancing therapeutic efficiency.[64] Chemical modification of exosomes is not limited to drug delivery. It can also be employed to deliver active exosomes, such as MSC-derived exosomes, whose surface can be linked with RVG peptide ligand to enhance their binding to the cortex and hippocampus.[65] These approaches showcase the versatility of chemical modification in tailoring exosome surfaces for various applications.

## TARGETING NON-PARENCHYMAL CELLS IN LIVER FIBROSIS

### Targeting HSCs in liver fibrosis

HSCs play a central role in liver fibrosis, and the regulation of HSC activation is crucial for antifibrotic effects. Stem cells, such as induced pluripotent stem cells (iPSCs) and HLSCs, have been shown to contribute to wound-healing responses and restoration of liver homeostasis following injury.[66] EVs derived from iPSCs have been found to modulate HSC activation and exhibit antifibrotic effects, which rely on transferring bioactive molecules such as miR-92a-3p, miR-10b-5p and miR-302-3p with potential antifibrotic properties. Treatment with iPSC-EVs results in decreased proliferation and chemotaxis of activated HSCs, important factors in fibrosis severity. Administration of murine iPSC-EVs in liver fibrosis models leads to downregulation of profibrogenic genes, including α-SMA, CollagenIα1 and TIMP-1.[67] HLSCs, a multipotent population of adult liver stem cells, also show potential in regulating HSC activation. Protein analysis showed the presence of 251 proteins in HLSC-EVs including transmembrane tyrosine kinase receptors, MMPs, some enzymes (glutathione reductase, creatine kinase and aspartate beta-hydroxylase) and transcription factors; and the most enriched pathways were related to cytokine



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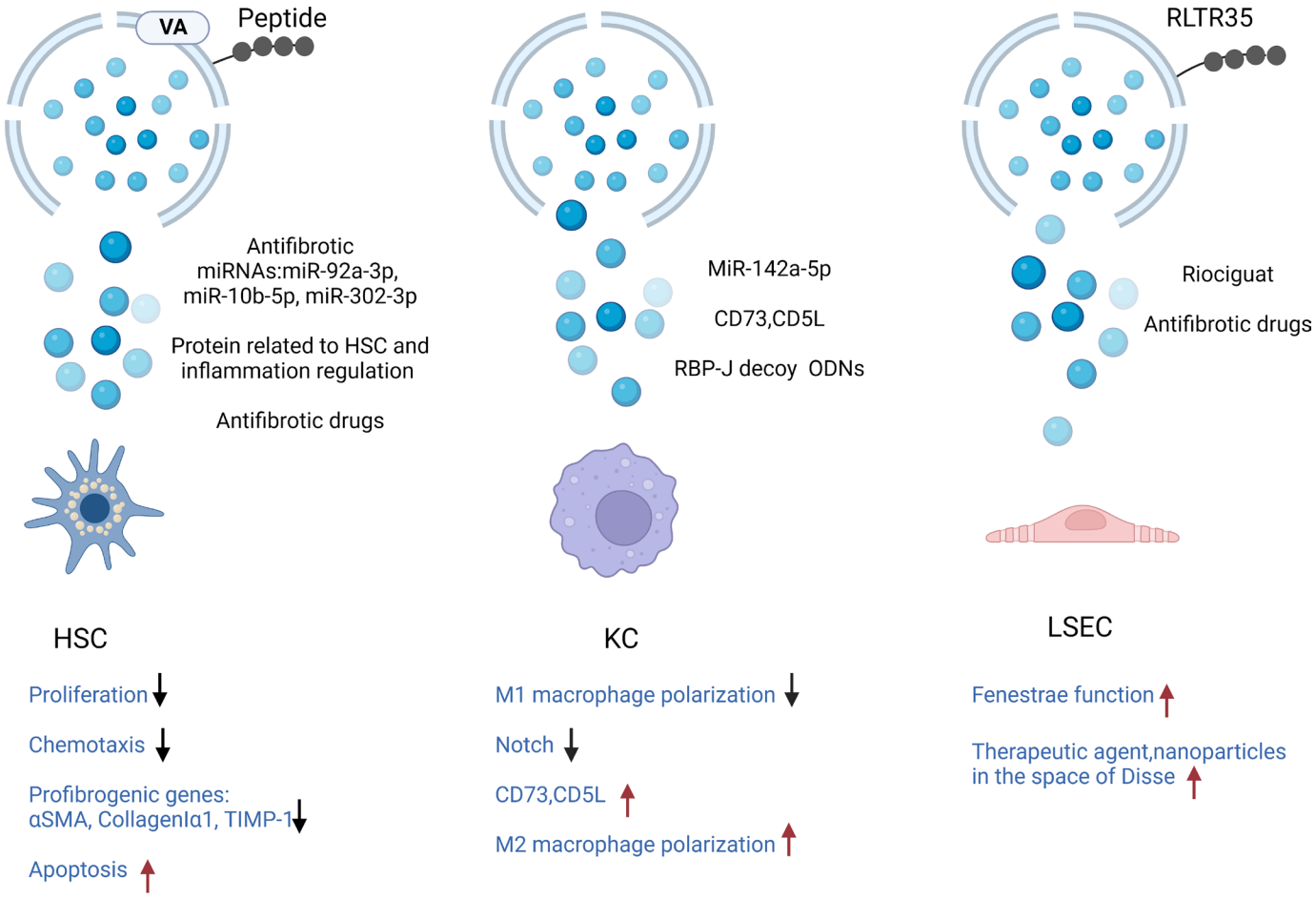


**Figure 4** Therapeutic effects of targeted extracellular vesicles (EVs) in non-parenchymal cells. After treatments of EVs, proliferation, chemotaxis and profibrogenic signals of activated HSCs decrease while expression of antifibrotic miRNA and apoptosis increase. As for KCs, their polarisation changes and expression of Notch declines while CD73 and CD5L raise. LSECs restore their fenestrae function and therapeutic agent or nanoparticles are allowed to access the space of Disse to play a role. HSC, hepatic stellate cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; ODN, oligodeoxynucleotide; SMA, smooth muscle actin. (Created with bioRender.com)

and inflammatory response, IL-10 anti-inflammatory activity, phosphatidylinositol 3-kinase pathways and the p53 pathway, which indicate that HLSC-EV protein cargo may modulate inflammation and improve liver fibrosis.[45] To achieve more targeted effects, EVs can be modified in various ways. For example, exosomes derived from human umbilical cord mesenchymal stem cells can be modified with a peptide named HSTP1, enabling precise treatment targeting activated HSCs in complex liver tissue.[68] Vitamin A-coupled small EVs (V-EVs) incorporate a vitamin A derivative into the membrane, allowing selective uptake by activated HSCs via receptor-mediated endocytosis. V-EVs demonstrate comparable antifibrotic effects to bare EVs at a lower dose.[69] In addition to stem cell-derived EVs, hepatocyte-derived EVs as well as EVs derived from natural killer cells have been shown to inhibit HSC activation and liver fibrosis.[70 71] Loading EVs with luteolin (LUT), a plant flavonoid with anti-inflammatory and antioxidant properties, leads to the suppression of HSC proliferation and induction of apoptosis.[72] As delivery vehicles, EVs possess natural biocompatibility, enabling efficient drug delivery. LUT-loaded EVs hold promise as effective nanocarriers for the treatment of liver fibrosis.

## Targeting KCs in liver fibrosis

KCs play a central role in the development and regression of hepatic fibrosis. They can activate HSCs and promote liver fibrosis by producing cytokines and chemokines. However, they also secrete MMPs to degrade and phagocytose existing ECM, thereby attenuating fibrosis.[73] KCs can switch between different phenotypes with opposing functions during fibrosis progression and remission. Therefore, therapeutic approaches targeting KCs aim to regulate their functional status rather than eliminate them.[74]

EVs derived from various stem cells have shown promise as therapeutic agents for liver fibrosis. For instance, EVs released by liver stem cells carry miR-142a-5p, which regulates macrophage polarisation through the miR-142a-5p/ctsb pathway, leading to improved fibrosis progression. This results in decreased markers of M1 macrophage polarisation and increased markers of M2 macrophage polarisation.[75] Gingival tissue-derived MSCs (GMSCs) are



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Table 2 Preclinical studies in liver fibrosis by targeting non-parenchymal cells

| Disease model | EV source | Route of administration | Mechanism(s) of action | Refs |
|---|---|---|---|---|
| CCl4-induced liver injury and fibrosis mice | iPSCs | Intravenous | Downregulation of α-SMA, Collagenlα1 and TIMP-1 | 67 |
| CCl4-induced liver injury and fibrosis rats | HSTP1-decorated Huc-MSC-EVs | Intravenous | Precise treatment targeting activated HSCs | 68 |
| TAA-induced liver fibrosis mice | Vitamin A-coupled MSC-EVs | Intravenous | Selective uptake by activated HSCs | 69 |
| CCl4-induced liver injury and fibrosis mice | NK cells | Intravenous | Decreased mRNA levels of α-SMA, CoL1A1, TGF-β1, TIMP-1 and PDGF-BB | 70 |
| CCl4 injury mice model | Normal mouse hepatocytes | Intraperitoneal | Reduced levels of α-SMA, CCN2 or Col1a1<br>Reduced hepatic monocyte (lyc6+) or macrophage (F4/80+) frequency | 71 |
| CCl4-induced liver injury and fibrosis rats | LUT-loaded MSC-EVs | Intraperitoneal | Decreased secretion of TNF-α, HP, TGF-β and MMP2 | 72 |
| CCl4-induced liver injury and fibrosis mice | LSCs | Intravenous | Upregulated the miR-142a-5p/ctsb pathway to regulate macrophage polarisation | 75 |
| TAA-induced chronic liver injury rat | ES-MSCs | Infused intrasplenically | Upregulation in MMP9 and MMP13, BCL-2, TGF-β1 and IL-10<br>Downregulation of Col1α, α-SMA, TIMP-1, BAX, TNF-α and IL-2 | 87 |
| CCl4 injury model rat | AMSCs | Intravenous | Decreased number of Kupffer cells and the mRNA expression levels of TNF-α, IL-1β, IL-6 and TGF-β<br>Suppressed LPS/ TLR4 signalling pathway | 88 |
| TAA-induced chronic liver injury mice | hIGF-I-loaded HUCPVCs-EVs | Intravenous | Decreased iNOS mRNA expression, IL-6 and TNF-α<br>Increased Arginase-1 | 79 |
| CCl4-induced liver injury and fibrosis rats | hAEC | Intravenous | Decreased percentage of F4/80+ liver infiltrating macrophages<br>Increased M2 macrophage density (F4/80+ CD206+) | 80 |
| BDL/CCl4-induced liver injury and fibrosis mice | HEK293T cells-EVs loaded with RBP-J decoy ODNs | Intravenous | Inhibited Notch signalling in macrophages<br>Decreased Hes1, IL-1β and IL-6<br>Upregulated Cyld | 82 |
| CCl4-induced liver injury and fibrosis mice | Synthesised nanoparticles with hyaluronic acid and SMV | Intravenous | Increased fenestrae with higher accumulation of CV-NPs/siCol1α1 in the space of Disse | 85 |
| ICR mice | Synthesised peptide-modified PEG-LPs | Intravenous | Increased efficiency of LECs targeting | 86 |

EVs, extracellular vesicles; HSC, hepatic stellate cell; Huc-MSC, human umbilical cord mesenchymal stem cell; IL, interleukin; iPSC, induced pluripotent stem cell; LUT, luteolin; MMP, matrix metalloprotein; NK, natural killer; ODN, oligodeoxynucleotide; PEG-LP, PEG-liposome; SMA, smooth muscle actin; TGF, transforming growth factor; TNF, tumour necrosis factor.

the mesenchymal stem cells of dental origin, with unique immunoregulatory capacity and secrete large amounts of EVs.[76] In a recent study, Watanabe *et al* found that EVs derived from GMSCs primed with a combination of two proinflammatory cytokines, TNF-α and interferon-α, synergistically promote anti-inflammatory M2 macrophage polarisation by increasing the expression of cluster of differentiation 73 (CD73) and CD5 molecule-like in EVs, which are mediated by mammalian target of rapamycin hypoxia-inducible factor-1α axis.[77] Moreover, EVs from human plasma, umbilical cord perivascular cells and amnion epithelial cells have been shown to ameliorate hepatic fibrogenesis by regulating macrophage function.[78–80]

As delivery vehicles, EVs can specifically target hepatic macrophages when administered via the tail vein. This enables them to deliver therapeutic cargo directly to KCs in the liver.[81] For example, EVs loaded with RBP-J decoy oligodeoxynucleotides inhibit Notch signalling in KCs and improve hepatic fibrosis when administered to mice via the tail vein.[82] These findings highlight the potential of EVs as targeted delivery vehicles for modulating macrophage function in the treatment of liver fibrosis.

## Targeting LSECs in liver fibrosis

During the development of liver fibrosis, the capillarisation of LSECs restricts substance exchange between the blood and the Disse space. This capillarisation accelerates HSC activation and fibrosis progression.[83] To address this issue, a systemic strategy for liver fibrosis treatment has been developed. The first step involves restoring the function of LSECs in therapeutic transport through the liver sinusoid.



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Two approaches have been explored for this purpose. The first approach involves using an sGC stimulator called riociguat or LSEC-targeting and fenestrae-repairing nanoparticles (HA-NPs/SMV). These interventions aim to repair the fenestrae (pores) in LSECs, allowing better access for antifibrosis agents or nanoparticles targeting HSCs to enter the Disse space. This can inhibit HSC proliferation, reduce collagen deposition and degrade deposited collagen.[84 85]

Targeted drug delivery systems have also been developed using modified peptides. For instance, a peptide-lipid conjugate has been designed to efficiently deliver molecules to LSECs. Peptide-modified PEG-liposomes (RLTR35) demonstrate potential for delivering drugs specifically to LSECs, offering a feasible approach for treating liver fibrosis.[86] These modified peptides enhance the specificity and effectiveness of drug delivery to LSECs, allowing for targeted treatment of liver fibrosis.

Although EVs targeting LSECs have hardly been reported, the thoughts above can be used in the development of them. For example, RLTR peptide attached EVs are supposed to target LSECs in theory and our team is trying to testify it and create EVs with LSECs targeting function.

Targeting non-parenchymal cells provides a promising treatment for liver fibrosis due to particular types of EVs working in targeted cells and finally to alleviate liver fibrosis (figure 4, table 2).

## CONCLUSION

Non-parenchymal cells, including HSCs, KCs and LSECs, play a significant role in the development of liver fibrosis. EVs derived from various sources have shown success in exerting antiapoptotic, anti-inflammatory and antifibrotic effects by targeting these non-parenchymal cells.

Compared with cell-based therapy, EV therapy possesses many advantages. EVs show good talent for going through biological barriers and carrying different cargoes. They also can be modified to improve their performance such as targeted ability. Moreover, EVs are easier to be gained and stored than cells owing to their rich source and stable character. However, to fully harness the therapeutic potential of EVs, further research is needed to understand their biogenesis, secretion and cellular entry mechanisms. Additionally, standards of EVs' clinical application should be made including the production, storage, transport, indication and adverse reaction of EVs. As for targeted treatment, exploring more therapeutic applications and optimising the targeting accuracy of EVs will be crucial to ensure their safety and efficacy as therapeutic agents or delivery vehicles for liver fibrosis treatment.

Overall, EVs hold great promise as a novel therapy for liver fibrosis, offering opportunities for targeted treatment and improved patient outcomes. It's a pity that there is no EV therapy for liver fibrosis under clinical trials up to now. So continued research and development in this field are indispensable to advancing the understanding and utilisation of EVs in liver fibrosis therapy.

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